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Versatile Synthesis of Head Group Functionalized Phospholipids via Oxime Bond Formation†

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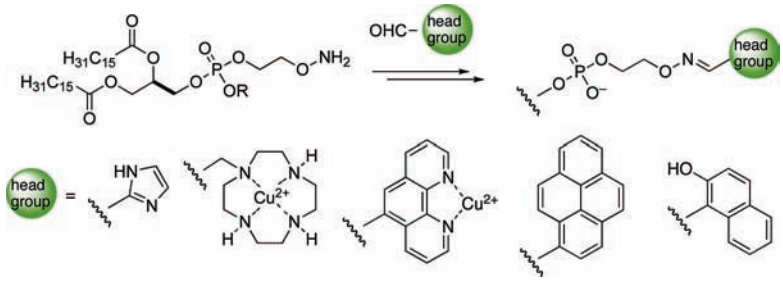
ABSTRACT



A method for introduction of various head groups on phospholipid frameworks via oxime bond formation has been developed for the synthesis of cyclen−Cu(II), pyrene, naphthalene, and other headgroup functionalized phospholipids that can cleave the membrane protein, hemagglutinin.

Artificial phospholipids have been employed as valuable molecular probes to investigate membrane related biosystems.[1] Furthermore, they have occupied a central position on supramolecular chemistry as useful building blocks to construct a nanoscale structure such as polymerizable liposome.[2] Among such artificial lipids, headgroup functionalized derivatives have been paid attention to as key molecules for membrane surface engineering,[3] target specific MRI,[4] siRNA delivery,[5] and protein crystallization.[6] Natural product,[7] peptide,[8] as well as photoactivatable functionality[9]−phospholipid conjugates at the headgroup have also been prepared. Although utility of headgroup-functionalized phospholipids has been recognized, a mild and efficient method to incorporate a functionalized headgroup onto a phospholipid framework has not yet been well exploited.

During the course of a previous investigation, we synthesized a phospholipid possessing EDTA−Fe(III) chelating headgroup and demonstrated its ability to cleave the influenza

† In memory of Professor Kiyoshi Tanaka, who passed away December 8, 2004.

‡ Kyoto University.

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(1) For examples, see: (a) Prestwich, G. D. *Acc. Chem. Res.* **1996**, *29*, 503. (b) Regen, S. L. *Curr. Opin. Chem. Biol.* **2002**, *6*, 729.

(2) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed.* **1988**, *27*, 113.

(3) (a) Grogan, M. J.; Kaizuka, Y.; Conrad, R. M.; Groves, J. T.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2005**, *127*, 14383. (b) Rabuka, D.; Forstner, M. B.; Groves, J. T.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 5947.

(4) Frias, J. C.; Williams, K. J.; Fisher, E. A.; Fayad, Z. A. *J. Am. Chem. Soc.* **2004**, *126*, 16316.

(5) Utku, Y.; Dehan, E.; Ouerfelli, O.; Piano, F.; Zuckermann, R. N.; Pagano, M.; Kirshenbaum, K. *Mol. BioSyst.* **2006**, *2*, 312.

(6) (a) Drakopoulou, E.; Tsivgoulis, G. M.; Mukhopadhyay, A.; Brisson, A. *Tetrahedron Lett.* **2000**, *41*, 4131. (b) Thompson, D. H.; Zhou, M.; Grey, J.; Kim, H. *Chem. Lett.* **2007**, *36*, 956.

(7) Matsuoka, S.; Matsumori, N.; Murata, M. *Org. Biomol. Chem.* **2003**, *1*, 3882.

(8) Cavalli, S.; Handgraaf, J.-W.; Tellers, E. E.; Popescu, D. C.; Overhand, M.; Kjaer, K.; Vaiser, V.; Sommerdijk, N. A. J. M.; Rapaport, H.; Kros, A. *J. Am. Chem. Soc.* **2006**, *128*, 13959.

(9) Wang, P.; Blank, D. H.; Spencer, T. A. *J. Org. Chem.* **2004**, *69*, 2693. See also, ref 1a.

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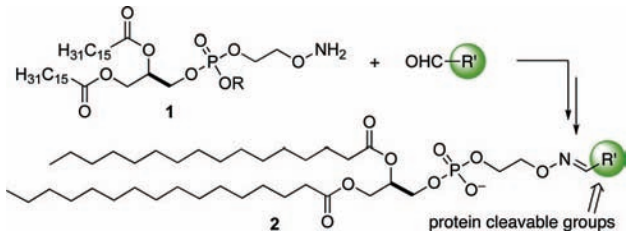
virus membrane protein, hemagglutinin (HA).[10] This work suggested that phospholipids could be a valuable platform for pursuing the affinity cleavage of integral membrane proteins. However, due to the requirement of headgroup introduction at an early stage of the synthesis, the synthetic route was not suitable for further derivative preparation.

Chemical cleavage of proteins is a valuable technology for investigating protein structures,[11] protein−protein interactions,[12] and also for degrading specific proteins and abrogating their functions.[13] However, such chemical cleavage has not yet expanded into the field of integral membrane protein research, although the methods to investigate the roles, functions, and structures of integral membrane proteins still require development.

This background motivated us to develop a general and convenient method to incorporate a functional group onto a phospholipid framework for preparing a series of derivatives, which would be beneficial for screening for more useful protein cleavable molecules. Here, we report the versatile synthesis of protein cleavable phospholipids **2**, by efficient introduction of protein cleavable head groups on **1** via oxime bond formation (oxime ligation)[14] at the latter stage of the synthetic route via the pivotal oxyamino functional group (Scheme 1). Additionally, we observed that these synthesized

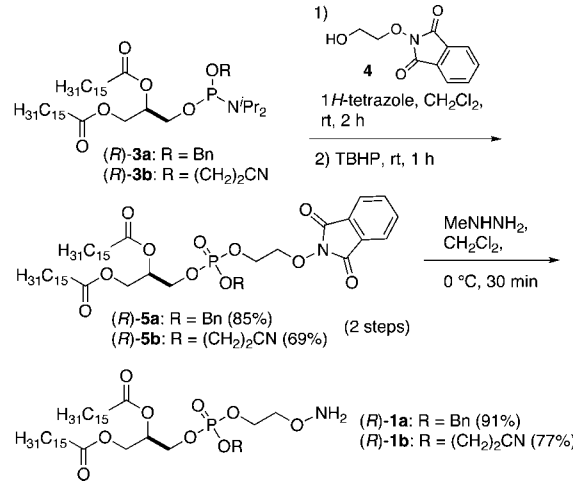
Scheme 1. Strategy for Synthesizing Artificial Phospholipids with Protein Cleavable Head Groups



phospholipids had protein cleavage activities within proteoliposomes.

First, phospholipid derivatives containing the oxyamino headgroup were prepared (Scheme 2). The amidite coupling between the phosphoroamidite **3a**[15] or **3b**[16] and the

Scheme 2. Preparation of the Oxyamino-Phospholipids



alcohol **4**,[17] which had a protected oxyamino group, proceeded smoothly in the presence of 1*H*-tetrazole, and successive oxidation of the phosphite intermediate by TBHP afforded the phosphotriesters **5a** and **5b** in 85 and 69% yields, respectively.

Removal of the phthalimide group of **5a**, by treatment with methyl hydrazine, yielded the desired *O*-benzyl-protected phosphodiester derivative **1a** in 91% yield. The same deprotection reaction afforded the *O*-cyanoethyl protected **1b** without any elimination of the cyanoethyl-protecting group (Scheme 2). Both of these protected phospholipids **1a** and **1b** worked well as key intermediates, in which the benzyl and cyanoethyl groups could be removed under catalytic hydrogenation and basic conditions, respectively, after introduction of the headgroup, via the formation of an oxime bond as shown in Table 1.

At first, we tested the introduction of imidazole groups, which have been used as catalytic centers for peptide bond hydrolysis[18] (Table 1, entries 1 and 2). Oxime bond formation between **1a** and 2-imidazole carboxyaldehyde (**6a**) proceeded smoothly in the mixed solvent system of $CHCl_3$−MeOH to give **7a** in 77% yield under neutral conditions without addition of any additive and/or catalyst (Table 1, entry 1). The isomeric 4-imidazole moiety was also incorporated into the cyanoethyl protected **1b** in excellent yield (Table 1, entry 2). The pyrene moiety, which has been reported to be a protein photocleavable group,[19] was also

(10) Furuta, T.; Sakai, M.; Hayashi, H.; Asakawa, T.; Kataoka, F.; Fujii, S.; Suzuki, T.; Suzuki, Y.; Tanaka, K.; Fishkin, N.; Nakanishi, K. *Chem. Commun.* **2005**, 4575.

(11) For example, see: Ermacora, M. R.; Ledman, D. W.; Fox, R. O. *Nat. Struct. Biol.* **1996**, *3*, 59.

(12) Datwyler, S. A.; Meares, C. F. *Trends Biochem. Sci.* **2000**, *25*, 408.

(13) (a) Jeon, J. W.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Song, J. B.; Suh, J. *Org. Lett.* **2002**, *4*, 4155. (b) Chae, P. S.; Kim, M.; Jeung, C.-S.; Lee, S. D.; Park, H.; Lee, S.; Suh, J. *J. Am. Chem. Soc.* **2005**, *127*, 2396. (c) Suzuki, A.; Tsumura, K.; Tsuzuki, T.; Matsumura, S.; Toshima, K. *Chem. Commun.* **2007**, 4260. (d) Suh, J.; Yoo, S. H.; Kim, M. G.; Jeong, K.; Ahn, J. Y.; Kim, M.; Chae, P. S.; Lee, T. Y.; Lee, J.; Lee, J.; Jang, Y. A.; Ko, E. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 7064.

(14) The oxime bond formation (oxime ligation) has been used to conjugate functional groups, such as fluorescent label, to biomolecules. See the following reference and references cited therein: Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581.

(15) Fukase, K.; Yoshimura, T.; Kotani, S.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 473.

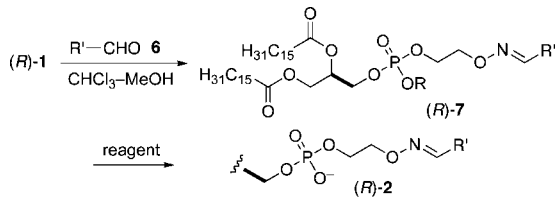
(16) Xu, Y.; Lee, S. A.; Kutateladze, T. G.; Sbrissa, D.; Shisheva, A.; Prestwich, G. D. *J. Am. Chem. Soc.* **2006**, *128*, 885.

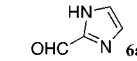
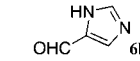
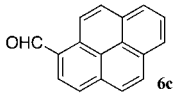
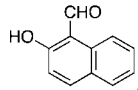
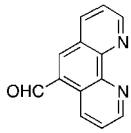
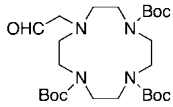
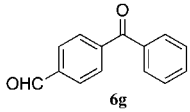
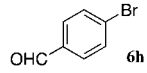
(17) Wey, S.-J.; Augustyniak, M. E.; Cochran, E. D.; Ellis, J. L.; Fang, X.; Garvey, D. S.; Janero, D. R.; Letts, L. G.; Martino, A. M.; Melim, T. L.; Murty, M. G.; Richardson, S. K.; Schroeder, J. D.; Selig, W. M.; Trocha, A. M.; Wexler, R. S.; Young, D. V.; Zemtseva, I. S.; Zifcak, B. M. *J. Med. Chem.* **2007**, *50*, 6367.

(18) Suh, J.; Oh, S. *J. Org. Chem.* **2000**, *65*, 7534.

(19) (a) Kumar, C. V.; Buranaprapuk, A. *Angew. Chem., Int. Ed.* **1997**, *36*, 2085. (b) Kumar, C. V.; Buranaprapuk, A.; Opiteck, G. J.; Moyer, M. B.; Jockusch, S.; Turro, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10361. (c) Kumar, C. V.; Buranaprapuk, A. *J. Am. Chem. Soc.* **1999**, *121*, 4262. (d) Buranaprapuk, A.; Kumar, C. V.; Jockusch, S.; Turro, N. J. *Tetrahedron* **2000**, *56*, 7019.

Table 1. Introduction of Functionalized Head Groups and Subsequent Deprotections



entry	lipid (**1**)	R'-CHO (**6**)	**7** (yield%)	reagent	**2** (yield%)
1	**1a**	 **6a**	**7a** (77)	Pd-C / H_2	**2a** (quant)
2	**1b**	 **6b**	**7b** (85)	*t*-BuNH$_2$	**2b** (quant)[b]
3	**1b**	 **6c**	**7c** (87)	*t*-BuNH$_2$	**2c** (quant)[b]
4	**1b**	 **6d**	**7d** (88)	*t*-BuNH$_2$	**2d** (quant)[b]
5	**1b**	 **6e**	**7e** (87)	*t*-BuNH$_2$	**2e** (quant)[b]
6	**1a**	 **6f**	**7f** (83)[a]	Pd-C / H_2	**2f** (quant)[a]
7	**1b**	 **6g**	**7g** (76)	*t*-BuNH$_2$	**2g** (quant)[b]
8	**1b**	 **6h**	**7h** (85)	*t*-BuNH$_2$	**2h** (quant)[b]

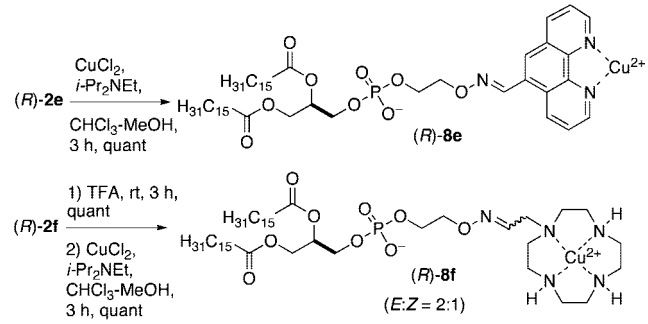
[a] Obtained as an *E*/*Z* mixture (*E*:*Z* = 2:1) of the oxime bond. [b] Obtained as a *t*-butylamine salt.

introduced (Table 1, entry 3). Besides 2-hydroxy-1-naphthaldehyde (**6d**), which is both a photocleavable protein and a metal-chelating group (Table 1, entry 4), the nitrogen-containing metal-chelating groups such as 1,10-phenanthroline (**6e**)[20] and the cyclen derivative **6f**[21] were also easily introduced, giving **7e** and **7f**, respectively, in excellent yields (Table 1, entries 5 and 6).

Subsequent deprotection of both the benzyl and cyanoethyl groups of these derivatives by catalytic hydrogenation and basic conditions, respectively, proceeded smoothly to yield the corresponding phospholipids **2a**−**2f** without any observable side reactions including reductive cleavage of the oxime bond. This headgroup introduction−deprotection sequence was readily applied to other artificial phospholipids such as the photoactivatable benzophenone derivative **2g**, which could be employed for photoaffinity labeling studies,[9] and the aryl bromide derivative **2h**, which could be used for further chemical modifications (Table 1, entries 7 and 8).

Next, the phenanthroline and cyclen modified phospholipids **2e** and **2f** were converted to their Cu-chelated forms[22] (Scheme 3). Treatment of **2e** with $CuCl_2$ in the presence of *i*-Pr_2NEt gave the desired Cu-chelated phenanthroline derivative **8e**. Removal of the Boc groups of the cyclen derivative **2f** with TFA treatment and successive Cu(II) chelation in the presence of *i*-Pr_2NEt gave the desired Cu-chelated cyclen phospholipid **8f** (Scheme 3).

Scheme 3. Preparation of Cu(II) Chelated Phospholipids



After producing these artificial phospholipids, we turned our attention to evaluating the ability of **2a**−**2d**, **8e**, and **8f** to cleave hemagglutinin (HA).[23]

These prepared phospholipids were relatively hydrophobic, so simple incubation of these phospholipids with influenza virus (A/WSN/33, H1N1) in MOPS buffer (pH 7.0) was ineffective for cleaving HA due to their insolubility. To overcome this difficulty, we reconstituted a membrane with the artificial phospholipids to place the phospholipids in close proximity to HA. The artificial phospholipids and HA were dissolved in a 5% octylglucoside solution, which was dialyzed against an external buffer solution to afford precipitates which included HA[24] when the phospholipids **2a**, **2c**, **2d**, and **8f** were used. A TEM image of the precipitate with **8f** clearly shows a liposome structure (50−150 nm in diameter), in which all of the surface is coated with cyclen−Cu(II) moieties (Figure 1a).

(20) Zaid, A.; Sun, J.-S.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Grierson, D. S.; Zain, R. *ChemBioChem* **2004**, *5*, 1550.

(21) For details of how the cyclen **6f** was prepared, see the Supporting Information.

(22) For protein cleavage by the 1,10-phenanthroline−Cu(II) complex, see: (a) Wu, J.; Perrin, D. M.; Sigman, D. S.; Kaback, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9186. (b) Galasco, A.; Crouch, R. K.; Knapp, D. R. *Biochemistry* **2000**, *39*, 4907. For protein cleavage by the cyclen−Cu(II) complex, see the following references and references cited therein: Jang, S. W.; Suh, J. *Org. Lett.* **2008**, *10*, 481.

(23) Wilson, I. A.; Skehel, J. J.; Wiley, D. C. *Nature* **1981**, *289*, 366.

(24) Determined by running SDS-PAGE on the precipitates from **2a**, **2c**, **2d**, and **8f** and visualizing bands for HA1 and HA2, both of which correspond to HA. We had difficulty applying the phenanthroline phospholipids **8e** to membrane reconstitution due to its insolubility in the 5% octylglucoside solution. See the Supporting Information.

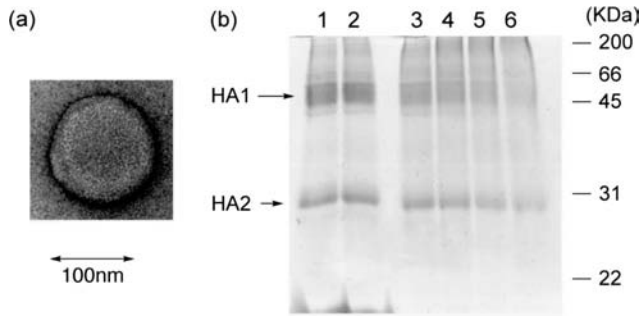


Figure 1. (a) TEM image of a proteoliposome with **8f**. (b) SDS-PAGE of the cleavage reactions with **8f** (coomassie-blue staining): gel shows proteoliposomes construced with phosphatidylcholine (lane 1, control) or with phosphatidylcholine incubated with Cu(II)−cyclen for 4 h (lane 2) or the reaction mixture immediately after initiation, and 1 h, 2 h, and 4 h later (lanes 3−6, respectively) with the proteoliposome **8f**.

The HA cleavage reactions were performed with these proteoliposomes. In the case of the proteoliposome **8f**, the hydrolytic cleavage of HA was initiated by changing the pH of the buffer from 7.0 (MOPS buffer) to 9.0 (boric acid buffer). The reaction was monitored by SDS-PAGE over 4 h (Figure 1b). SDS-PAGE of the liposomes immediately after cleavage initiation shows the HA1 and HA2 bands, both of which correspond to HA (Figure 1b, lane 3).[25] After a 2 h incubation at 50 °C, a decrease in the HA bands (HA1 and HA2) was observed (Figure 1b, lane 5). Moreover, after a 4 h incubation, they had almost completely disappeared (Figure 1b, lane 6). These band decreases clearly indicated that HA was being cleaved.[26,27] It is noteworthy that no cleavage of HA was observed when the cyclen−Cu(II) complex was added to the proteoliposomes made with phosphatidylcholine under the same reaction conditions (Figure 1b, lane 2).

Although the imidazole-tethered phospholipid **2a** did not cleave HA, the pyrene and 2-hydroxynaphthalene modified phospholipids **2c** and **2d** did exhibit cleavage activities under photoirradiation conditions with UV light at 365 nm and degraded HA within 5 and 30 min, respectively.[28,29] The efficient cleavage by **8f**, **2c**, and **2d** clearly indicates that these proteoliposomes provide excellent reaction environments for integral membrane protein cleavage, in which the active site of the cleavage reactions is in close proximity to the proteins. It also indicates that the phospholipid structure is a promising platform for the development of further protein-specific scissor molecules.

In conclusion, we have developed a mild and convenient way to access a variety of headgroup functionalized phospholipids. We are currently modifying the phospholipid structure further toward site-selective as well as molecule-specific cleavage of integral membrane proteins.

Acknowledgment. We thank Prof. Koji Nakanishi and Drs. Nathan Fishkin and Craig Parish (Columbia University) for their valuable discussions. Financial support was provided by JSPS and a Grant-in Aid for Young Scientists (B) 17710187.

Supporting Information Available: Detailed experimental procedures and spectroscopic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(25) Selimova, L. M.; Zaides, V. M.; Zhdanov, V. M. *J. Virol.* **1982**, *44*, 450.

(26) Staining with biotin−ConA also decreased the HA1 band. Cleaved HA fragments were visualized as smears by immunostaining with anti-P50 (H1N2) rabbit polyclonal antibody. See Supporting Information. For anti-P50, see: Suzuki, T.; Takahashi, T.; Guo, C.-T.; Hidari, K. I.-P. J.; Miyamoto, D.; Goto, H.; Kawaoka, Y.; Suzuki, Y. *J. Virol.* **2005**, *79*, 11705.

(27) The oxidative conditions were extremely powerful for HA cleavage. HA was almost completely cleaved within 3 min at 0 °C after addition of H_2O_2 and sodium ascorbate. See Supporting Information.

(28) For the cleavage of HA with **2c** and **2d**, see Supporting Information.

(29) Pyrene and 2-hydroxynaphthalene themselves did not show cleavage activity. See Supporting Information.